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Rescue of polyglutamine-mediated cytotoxicity by double-stranded RNA-mediated RNA interference

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RNA interference (RNAi) is a mechanism that appears to control unwanted gene expression in a wide range of species. In *Drosophila*, RNAi is most effectively induced by double-stranded RNAs (dsRNAs) of over ~80 nucleotides (nt) and in mammalian cells an RNAi-like inhibition of gene expression has been shown to be mediated by dsRNAs of ~21–23 nt. To test if RNAi can be used to specifically down-regulate a human disease-related transcript we have used *Drosophila* and human tissue culture models of the dominant genetic disorder spinobulbar muscular atrophy (SBMA). A variety of different dsRNAs were assessed for the ability to inhibit expression of transcripts that included a truncated human androgen receptor (*ar*) gene containing different CAG repeat lengths (16–112 repeats). In *Drosophila* cells, dsRNAs corresponding to non-repetitive sequences mediated a high degree of sequence-specific inhibition, whereas RNA duplexes containing CAG repeat tracts only induced gene-specific inhibition when flanking *ar* sequences were included; dsRNAs containing various lengths of CAG repeats plus *ar* sequences were unable to induce allele-specific interference. In mammalian cells we tested sequence-specific small dsRNAs of 22 nt; these rescued the toxicity and caspase-3 activation induced by plasmids expressing a transcript encoding an expanded polyglutamine tract. This study demonstrates the feasibility of targeting a transcript associated with an important group of genetic diseases by RNAi.

INTRODUCTION

Gene silencing mediated by double-stranded RNAs (dsRNAs) has emerged as an important mechanism regulating exogenous and potentially endogenous gene expression. Termed post-transcriptional gene silencing in plants and fungi, and RNA interference (RNAi) in other organisms (1), this process is mediated by dsRNA and induces the sustained down-regulation of the target gene corresponding to the dsRNA (2,3) as a result of degradation of the target mRNA. Whereas most work to date has been performed in lower eukaryotes, there is also evidence for RNAi in mouse zygotes and embryos (4,5) and in both mouse and human cells in culture where dsRNAs of ~21–23 nucleotides (nt) have been shown to specifically inhibit gene expression (6,7). Because of the efficacy and ease with which RNAi can be applied, this mechanism has been rapidly exploited in *Caenorhabditis elegans* and *Drosophila* as a reverse genetics tool (8–10). The genetic treatment of a number of disease states including infectious disease, tumor development and dominant genetic disorders would be another application for a mechanism that specifically down-regulates gene expression. Previous methodologies that have been exploited to achieve this goal have included antisense technology and catalytic ribozymes (11); however, the efficacy of these approaches has been mixed, particularly *in vivo*. Given the effectiveness of dsRNA-mediated gene silencing, we wanted to assess whether this mechanism could be used to specifically down-regulate a human disease-related transcript.

At least eight human neurodegenerative disorders, including Huntington's disease (HD) and spinobulbar muscular atrophy (SBMA) (Kennedy's disease) are caused by expansion of trinucleotide (CAG) repeats (12). SBMA is an X-linked, adult onset motor neuronopathy caused by a progressive loss of lower motor neurons in the spinal cord and brain stem and sensory neurons in the dorsal root ganglia. The genetic defect underlying SBMA is an expansion of a polymorphic trinucleotide CAG repeat in the first exon of the androgen receptor (*ar*) gene. The normal range of repeats is 11–35 glutamines; individuals with SBMA have an expanded CAG repeat length encoding an increased polyglutamine (polyQ) tract of 38–62 residues (13). The androgen receptor (AR) is a ligand-activated transcription factor and its biological functions are well characterized. However, the physiological functions of the polyQ

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tract in the N-terminal transactivation domain of the protein, as well as the mechanism by which this expansion leads to neurodegeneration, are unknown, though most evidence suggests that polyglutamine expansion in the AR protein results in a toxic gain-of-function.

Aggregation of mutant polyQ proteins is a feature present in all of the polyglutamine disorders (14). In individuals with SBMA, intranuclear aggregates that contain the AR protein are present in motor neurons of the brain stem and spinal cord as well as in other tissues that normally express this protein (15,16). Similar findings have been reported in transgenic mouse models of HD and SBMA and patients with HD (17–20). These protein aggregates are insoluble, and in cell culture are associated with abnormal proteolytic processing of the AR protein (21–23). Though protein mis-folding and aggregation is seen in most of the polyQ diseases there is still some debate as to whether aggregate formation is directly neurotoxic or a by-product of disease progression. Some studies have suggested that protein aggregation or nuclear inclusions are neither necessary nor sufficient to initiate disease (24–26); however, there is also evidence that peptides containing expanded polyQ tracts are intrinsically cytotoxic (27–31). There are several mechanisms by which these abnormal proteins could be acting pathogenically, including, sequestration of critical cellular factors into protein aggregates, inhibition of key cellular pathways such as ubiquitination, and the induction of proteolysis. Evidence for the last of these possible mechanisms is increasing with the observations by several groups of an association between the expression of proteins containing expanded polyQ tracts and caspase (cysteine aspartate-specific proteases)-dependent apoptotic cell death (32–37).

Though our understanding of the molecular basis of these progressive disorders has improved in recent years, new strategies to modulate the effect of these toxic proteins have been more difficult to develop. Beyond symptomatic treatment, a majority of recent studies investigating potential therapeutic approaches to polyQ disorders have concentrated on suppressing protein aggregation or inhibiting apoptosis. Several studies have shown that co-expression or over-expression of heat shock chaperone proteins that are critical for protein folding can inhibit the toxic effects of the polyQ proteins (38–41) and studies inhibiting caspase-associated cell death have shown that blocking caspase-8 recruitment into polyQ protein aggregates significantly reduces cell death (42). In addition, inhibiting capase cleavage of the huntingtin protein delays disease onset in transgenic mice (43) and in cell culture (37). An alternative approach to targeting a mutant polyQ protein is to down-regulate its mRNA. Two principal methods have been reported to specifically trigger the degradation of a target transcript: one makes use of antisense oligonucleotides or transcripts and the other uses synthetic ribozyme sequences. Antisense oligonucleotides or transcripts down-regulate gene expression by translational blockade and induction of RNA degradation mainly through the action of the RNase H ribonuclease (11,44). Ribozymes have an enzymatic capability that specifically binds and digests RNA at a defined site (45,46). Although *in vitro* data has been encouraging, the application of these methods has been difficult due to the poor *in vivo* instability of antisense oligonucleotides and the limitation of finding a suitable target sequence for a given ribozyme. Studies aimed at down-regulating transcripts associated with dominant genetic disorders have included application of antisense technology to Marfan's syndrome (47) and HD (48), and the use of catalytic ribozymes to target rhodopsin transcripts that are associated with autosomal dominant retinitus pigmentosa (49,50). This study now shows the feasibility of targeting an RNA species from an important group of genetic diseases by dsRNA triggered RNAi, including rescue of the caspase-dependent apoptotic cell death induced by polyQ expansions in human cells.

RESULTS

Gene-specific dsRNAs can interfere with expression from *arcag$_n$gfp* fusion transcripts in *Drosophila* S2 cells

Drosophila embryonic S2 cells have been used extensively as a cell culture system to analyze the RNAi mechanism and its application (51–53). Though several transgenic *Drosophila* models of polyQ diseases have been developed (31,54–56), no cell culture model has been described. To develop a *Drosophila* cell culture model of SBMA, *Drosophila* expression plasmids were constructed to express fusion transcripts containing portions of the human *ar* gene, with CAG tracts of 26, 43 or 106 repeats and the marker green fluorescent protein gene (*gfp*) (Fig. 1A). Northern analysis of mRNA purified from S2 cells transfected with each expression plasmid showed appropriately sized *gfp* containing transcripts (Fig. 3F and data not shown).

Transient transfection of S2 cells with plasmids containing 43 and 106 CAG trinucleotide repeats showed GFP-positive protein aggregates and aggresomes (Fig. 1B). Low-level GFP-associated protein aggregation was observed in S2 cells transfected with pAct.ARCAG43GFP after ~2–3 days; localized aggresomes were seen in 4–5 days. S2 cells transfected with pAct.AR.CAG106GFP formed GFP-positive protein aggregates and aggresomes within 24 h. Of those S2 cells transfected, ~4% of pAct.ARCAG43GFP and 35% of pAct.ARCAG106GFP-transfected cells developed GFP-positive protein aggregates 3 days after transfection; this increased to 10% of pAct.ARCAG43GFP-transfected cells and 90% of pAct.ARCAG106GFP-transfected cells 5 days after transfections. These results are consistent with observations in mammalian cells, where trinucleotide length has been shown to correlate with the degree of protein aggregation. Trinucleotide repeats in the range of 40–50 produce proteins that are susceptible to aggregation but this develops over a longer period of time than when a larger repeat expansion is used, represented in this study by the 106 CAG repeat construct (22,23,57). No protein aggregation was seen when the control pAct.GFP or pAct.ARCAG26GFP plasmids were used.

To assess the effect of gene-specific dsRNAs on the expression of the fusion transcripts from each of the pAct.ARCAG$_n$GFP plasmids dsRNAs of ~80 nt corresponding to portions of the *gfp* gene and the *ar* gene were generated using the appropriate sense and antisense oligomers (Table 1 and Fig. 2); dsRNAs corresponding to the β-galactosidase gene (*LacZ*) and the chloramphenicol acetyl transferase (CAT) gene (*cat*) were used as controls. *Drosophila* S2 cells were co-transfected with each dsRNA molecule and each of the *Drosophila* expression plasmids. Cells were harvested 72 h after initiation of the transfection and assayed for GFP expression (Fig. 3). In all cases the

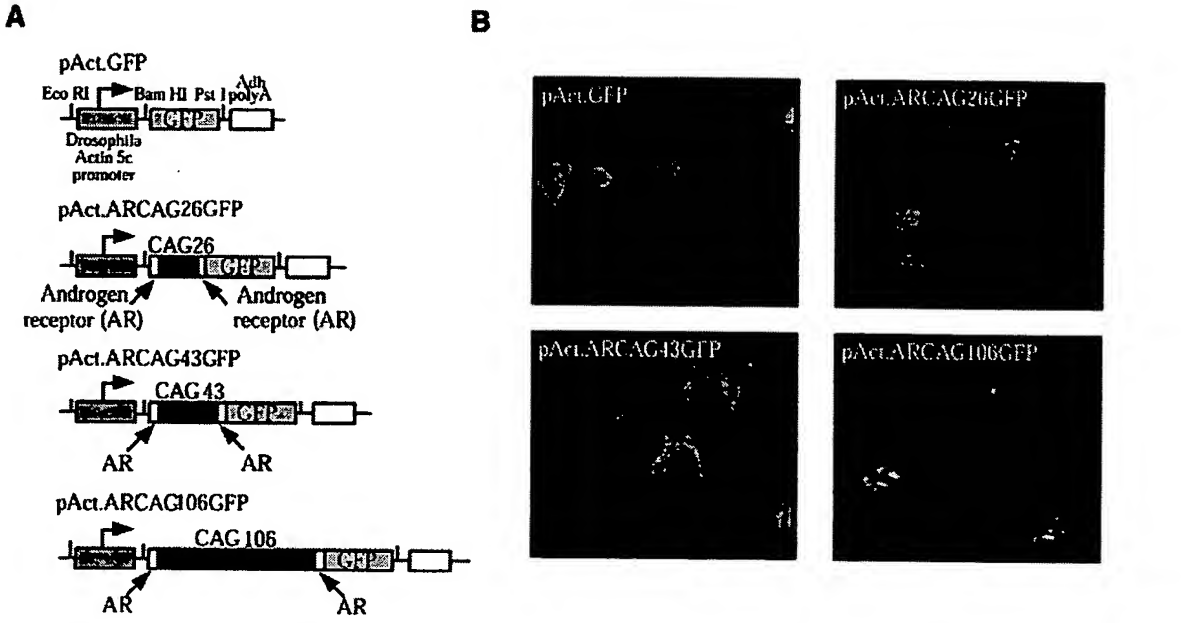

Figure 1. *Drosophila* plasmids expressing *arcag*$_n$*gfp* fusion transcripts and their transient expression in S2 cells. (A) Diagrammatic representations of *Drosophila* expression plasmids used in this study. (B) S2 cells (2×10^6) were transfected with pAct.GFP, pAct.ARCAG26GFP, pAct.ARCAG43GFP and pAct.ARCAG106GFP (2 μg of plasmid DNA) and were analyzed by deconvolution microscopy 72 h after initiation of transfection. GFP fluorescence is in green and nuclei are counter stained in blue using Hoeschst staining.

gfp dsRNA specifically inhibited expression of GFP, including GFP expression from all three of the pAct.ARCAG$_n$GFP fusion plasmids. The *gfp* dsRNA inhibited GFP expression from pAct.ARCAG26GFP by ~75%, GFP expression from pAct.ARCAG43GFP by ~80% and GFP expression from pAct.ARCAG106GFP by ~85%. The 80% inhibition of GFP expression from the pAct.GFP plasmid is consistent with our previous data (51).

The *ar* dsRNA specifically inhibited GFP expression from the fusion transcripts containing human AR sequences. The *ar* dsRNA induced significant (60–80%) down-regulation of GFP expression from all three of the pAct.AR(CAG)$_n$GFP fusion plasmids (Fig. 3A–C). The *ar* dsRNA had no effect on GFP expression from the parental plasmid pAct.GFP, which contains no *ar* sequences (Fig. 3D), and only the dsRNA corresponding to *cat* inhibited CAT expression; the *ar* dsRNA had no significant effect on CAT expression (Fig. 3E).

Northern analysis of mRNA purified from *Drosophila* cells treated with these dsRNAs also demonstrates gene-specific inhibition with a correlation between the number of *gfp* and *ar* containing transcripts and the level of GFP expression observed (Fig. 3F). Using phosphoimage analysis, the levels of the *gfp*-positive and *ar*-positive signals were first normalized using the *Drosophila* glyceraldehyde 3-phosphate dehydrogenase-1 (*gapdh-1*) hybridization signal; the relative levels of the *ar*/*gfp*-positive transcripts were then compared. The *gfp* dsRNA induced a 70–90% decrease in the *ar*/*gfp*-positive transcript. The *ar* dsRNA induced a 40% decrease in the level of the *arcag26gfp* transcript; the *arcag43gfp* and *arcag106gfp* mRNA levels were reduced by 60–80% relative to controls.

The effect of dsRNAs containing CAG repeats on transgene expression in *Drosophila* S2 cells

Several studies of RNAi in invertebrate systems have considered the size effects and sequence content of the input dsRNA used to mediate this process (51,58–61), but a transcript containing different numbers of CAG repeats flanked by complex human sequences represents a novel target. To examine the effect of dsRNAs containing highly repetitive sequences, we used dsRNA consisting of 27 CAG repeats (*cag27*) and 27 CAG repeats plus 21 nt from the 5′ end of the human AR sequence (*arcag27*) (Table 1 and Fig. 2). Unlike the specific effect seen with the use of complex gene-specific dsRNAs such as the *ar* and *gfp* dsRNAs, the *cag27* dsRNA induced a non-specific inhibition of transgene expression in *Drosophila* cells. Representative data shown in Figure 4 shows the significant inhibition (60–75%) of GFP expression from the pAct.ARCAG$_n$GFP fusion plasmids (Fig. 4A-C); however, we also consistently saw a decrease in the level of GFP expression from the parental pAct.GFP plasmid of the order of 30–40% (Fig. 4D). We tested a number of preparations of the *cag27* dsRNA, but all had the same effect on GFP expression from pAct.GFP. The addition of 21 nt of the *ar* sequence immediately upstream of the CAG repeat did not improve the specificity of this inhibition, as the *arcag27* dsRNA also reduced GFP expression from the pAct.GFP plasmid by ~50% (Fig. 4D). GFP expression from the fusion transcripts was inhibited by 70–80% by the *arcag27* dsRNA (Fig. 4A–C). Interestingly, this non-specific interference with transgene expression could, in large part, be alleviated by the use of

Table 1. Single-stranded oligoribonucleotides used to form dsRNAs

ssRNA	Sequence
lacZ s 81 nt	5′ r(agc ucc ugc acu gga ugg ugg cgc ugg aug gua agc cgc ugg caa gcg gug aag ugc cuc ugg aug ucg cuc cac aag gua) 3′
lacZ as 81 nt	5′ r(uac cuu gug gag cga cau cca gag gca cuu cac cgc uug cca gcg gcu uac cau cca gcg cca cca ucc agu gca gga gcu) 3′
cat s 78 nt	5′ r(cau cgc ucu gga gug aau acc acg acg auu ucc ggc agu uuc uac aca uau auu cgc aag aug ugg cgu guu acg gug) 3′
cat as 78 nt	5′ r(cac cgu aac acg cca cau cuu gcg aau aua ugu gua gaa acu gcc gga aau cgu cgu ggu auu cac ucc aga gcg aug) 3′
gfp s 78 nt	5′ r(cua cgu cca gga gcg cac cau cuu cuu caa gga cga cgg caa cua caa gac ccg cgc cga ggu gaa guu cga ggg cga) 3′
gfp as 78 nt	5′ r(ucg ccc ucg aac uuc acc ucg gcg cgg guc uug uag uug ccg ucg ucc uug aag aag aug gug cgc ucc ugg acg uag) 3′
ar s 78 nt	5′ r(ag ggu gag gau ggu ucu ccc caa gcc cau cgu aga ggc ccc aca ggc uac cug guc cug gau gag gaa cag caa ccu u) 3′
ar as 78 nt	5′ r(a agg uug cug uuc cuc auc cag gac cag gua gcc ugu ggg gcc ucu acg aug ggc uug ggg aga acc auc cuc acc cu) 3′
cag27 s 81 nt	5′ r(cag cag cag cag cag cag cag cag cag cag cag cag cag cag cag cag cag cag cag cag cag cag cag cag cag cag cag) 3′
cag27 as 81 nt	5′ r(cug cug cug cug cug cug cug cug cug cug cug cug cug cug cug cug cug cug cug cug cug cug cug cug cug cug cug) 3′
arcag27 s 104 nt	5′ r(ggc gcc agu uug cug cug cug cag cag cag cag cag cag cag cag cag cag cag cag cag cag cag cag cag cag cag cag cag cag cag cag cag cag cag cag cag cag ca) 3′
arcag27 as 104 nt	5′ r(ug cug cug cug cug cug cug cug cug cug cug cug cug cug cug cug cug cug cug cug cug cug cug cug cug cug cug cug cug cug cug cug cug cug cug cug cug cug acu ggc gcc) 3′
cat s 22 nt	5′ PO_4 r (gag uga aua cca cga cga uuu c) 3′ OH
cat as 22 nt	5′ PO_4 r(aau cgu cgu ggu auu cac ucc a) 3′ OH
gfp s 22 nt	5′ PO_4 r(gca agc uga ccc uga agu uca u) 3′ OH
gfp as 22 nt	5′ PO_4 r(gaa cuu cag ggu cag cuu gcc g) 3′ OH
5′ar s 22 nt	5′ PO_4 r(gcu caa gga ugg aag ugc agu u) 3′ OH
5′ar as 22 nt	5′ PO_4 r(cug cac uuc cau ccu uga gcu u) 3′ OH
3′ar s 22 nt	5′ PO_4 r(cug gau gag gaa cag caa ccu u) 3′ OH
3′ar as 22 nt	5′ PO_4 r(ggu ugc ugu ucc uca ucc agg a) 3′ OH

The *lacZ* 81 nt, *cat* 78 nt and *gfp* 78 nt oligoribonucleotide sequences have been reported previously by Caplen *et al.* (51), as have the *cat* and *gfp* 22 nt sequences (7). s, sense; as, antisense.

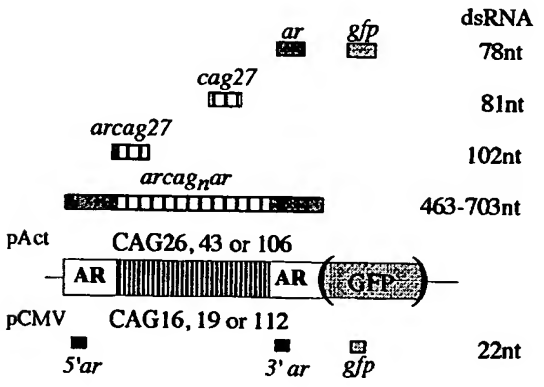

Figure 2. A diagrammatic representation of the dsRNAs used in this study related to the *arcag$_n$(±gfp)* fusion transcript.

larger regions of *ar* sequence on both sides of the CAG repeat tract. This restoration of specificity was irrespective of the size of the intervening CAG tract (Fig. 4E–H). However, though these dsRNAs showed sequence specificity, in that they had minimal effect on transgene expression from the parental pAct.GFP plasmid, we saw no evidence for allelic specificity, that is, dsRNAs with different CAG repeat lengths (26, 43 or 106) interfered with expression from all three of the *arcag$_n$gfp* transcripts irrespective of the number of repeats in the target transcript (Fig. 4E–G). The interference mediated by the *arcag$_n$ar* dsRNAs was of a similar level to that seen using the 78 nt *gfp* dsRNA (Fig. 4E–H).

Rescue of cell toxicity induced by transcripts expressing expanded polyQ repeats by small dsRNAs in human cells

In mammalian cells, dsRNAs of over ~80 nt can trigger a variety of cellular responses that result in a non-specific decrease in gene expression and frequently cell death (62). However, recently, we and others have shown that small dsRNAs of ~21–23 nt can mediate a sequence-specific inhibition of gene expression in mouse and human cells (6,7). To determine whether small dsRNAs can specifically interfere with expression of a polyQ protein and thus rescue the increased cell death associated with an expanded CAG tract we co-transfected human HEK-293T cells with mammalian expression plasmids expressing a fusion transcript consisting of a truncated version of the human AR containing either 112 or 19 CAG repeats and *gfp* (pCMV.ARCAG112GFP and pCMV.ARCAG19GFP, respectively) and 22 nt dsRNAs corresponding to either *cat* or *gfp* (Table 1 and Fig. 2). The small dsRNAs used were designed with 5′ PO_4 and 2 nt 3′

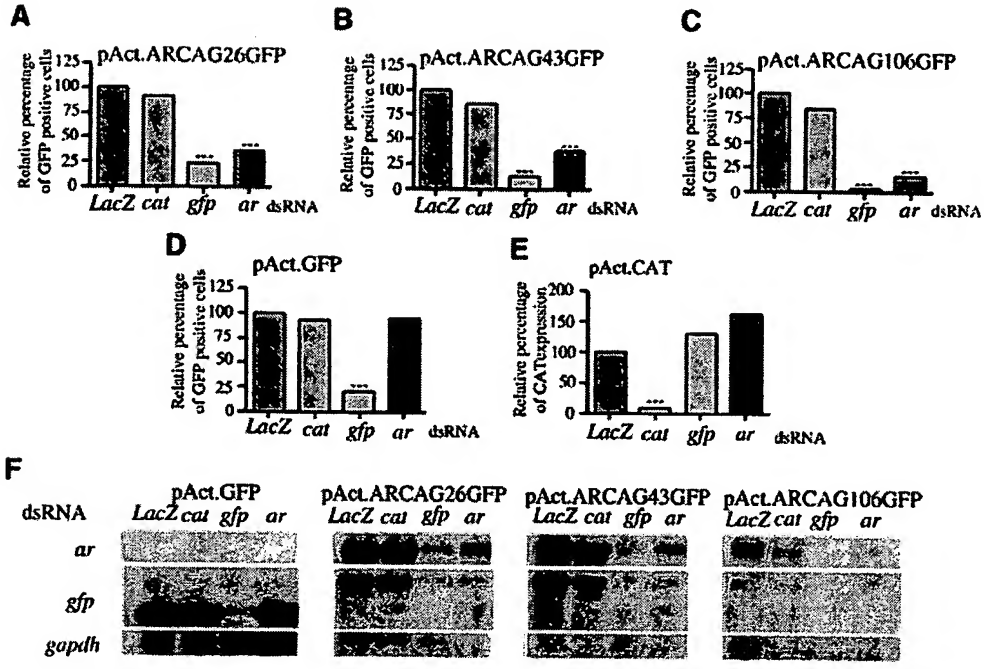

Figure 3. Inhibition of transgene expression by gene-specific dsRNA molecules. dsRNA molecules corresponding to *LacZ*, *cat*, *gfp* or *ar* were co-transfected with either (A) pAct.ARCAG26GFP, (B) pAct.ARCAG43GFP, (C) pAct.ARCAG106GFP, (D) pAct.GFP or (E) pAct.CAT. S2 cells (2×10^6) were transfected with 2 μg of plasmid DNA and 1 μg of dsRNA; cells were analyzed 72 h after initiation of transfection. Data shown is representative of at least three independent experiments using each dsRNA and is normalized to the levels of GFP expression observed in those cells co-transfected with the plasmid shown and the control *LacZ* dsRNA, $n = 3$ for each plasmid/dsRNA combination. (F) Northern analysis of poly(A)$^+$ RNA (~2 μg of each sample) purified from S2 cells transfected with pAct.GFP, pAct.ARCAG26GFP, pAct.ARCAG43GFP and pAct.ARCAG106GFP and either *LacZ*, *cat*, *gfp* or *ar* dsRNA. The northern blot was sequentially hybridized with (i) human *ar*, (ii) *gfp* and (iii) *Drosophila gapdh-1*-specific DNA probes. Statistical analysis was performed by comparison to *LacZ* dsRNA-transfected cells. $^{**}P < 0.01$, $^{***}P < 0.001$.

overhangs, a structure that is characteristic of the RNase III (Dicer) enzymatic cleavage that has been associated with RNAi (63). We assayed for GFP expression by deconvolution microscopy (Fig. 5A–F) and assessed toxicity using a fluorescence-activated cell-sort analysis (FACS)-based viability assay of cell death (Fig. 5G and H) 96 h after initiation of transfection. Consistent with previous observations (7), a 22 nt *gfp* dsRNA inhibited GFP expression from both fusion transcripts (Fig. 5E and F); a 22 nt dsRNA corresponding to *cat* had no effect on GFP expression. Further, protein aggregates were clearly evident in pCMV.ARCAG112GFP/*cat* 22 nt dsRNA co-transfected cells whereas there was little or no evidence of protein aggregation in those cells transfected with pCMV.ARCAG112GFP and the 22 nt *gfp* dsRNA. Interference with expression from the ARCAG112GFP transcript mediated by the 22 nt *gfp* dsRNA also significantly reduced the cell death associated with the presence of a polyQ expansion (Fig. 5G) towards control levels (Fig. 5H).

We next tested two 22 nt dsRNAs corresponding to different regions of the human *ar* gene; one region was 5′ of the CAG repeat (*5′ar*) and the second target region was 3′ of the CAG repeat (*3′ar*). Both dsRNAs significantly reduced the cytotoxic effect mediated by the presence of an AR protein with an expanded polyQ tract (Fig. 5I), with the 22 nt *5′ar* dsRNA inducing the greatest level of rescue by reducing the percentage of dead cells to the range seen in control transfections (Fig. 5J). Importantly, both 22 nt *ar* dsRNAs also significantly reduced the levels of caspase-3 activity induced by the ARCAG112 protein. The control *cat* 22 nt dsRNA had no effect on caspase-3 activity indicating the specificity of the effect mediated by the *ar* 22 nt small dsRNAs.

DISCUSSION

dsRNA-mediated silencing of gene expression is increasingly being seen as an important epigenetic mechanism controlling gene expression and as a reverse genetics tool (8,64–66). With the recent description of an RNAi-like mechanism in mammalian somatic cells (6,7) it may now also be possible to eventually exploit dsRNA triggered RNAi as a therapeutic approach akin to current strategies that exploit single-stranded antisense oligonucleotides or transcripts to down-regulate gene expression. In this study, we first assessed the feasibility of such an approach in a model system where RNAi has been relatively well characterized by developing a cell culture model of the dominant genetic disorder SBMA (Kennedy's disease) in *Drosophila* S2 cells. We then used the recently described process of small dsRNA (21–23 nt)-triggered RNAi in mammalian cells to assess whether this could be used to rescue the toxic effects of a truncated human AR protein containing

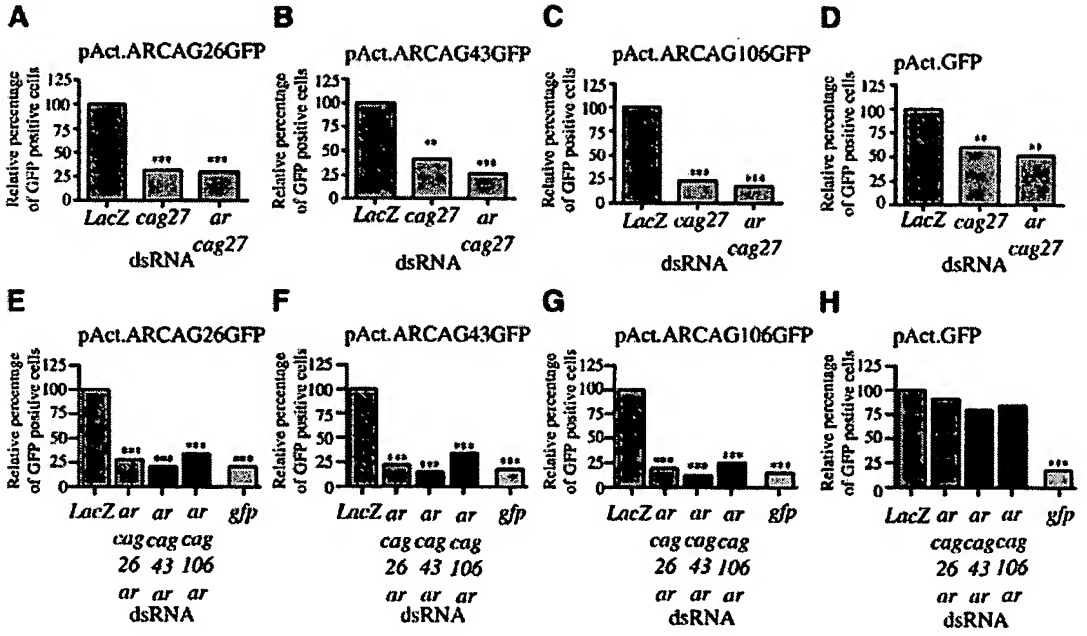

Figure 4. The effect of CAG containing dsRNA molecules on the expression of *arcag$_n$gfp* fusion transcripts. S2 cells were transfected with pAct.ARCAG26GFP (A and E), pAct.ARCAG43GFP (B and F), pAct.ARCAG106GFP (C and G) or pAct.GFP (D and H) and dsRNA molecules corresponding to: *LacZ* (A–H), 27 CAG trinucleotide repeats (*cag27*) (A–D), 21 nt from the upstream flanking sequence of the human AR plus 27 CAG repeats (*arcag27*) (A–D), *arcag26ar* (E–H), *arcag43ar* (E–H), *arcag106ar* (E–H) or *gfp* (E–H). S2 cells (2×10^6) were transfected with 2 μg of plasmid DNA and 0.5–1 μg of dsRNA; cells were analyzed 72 h after initiation of transfection, (A–D) $n = 3$, (E–H) $n = 5$, data shown is normalized to the levels of GFP expression observed in those cells co-transfected with the plasmid shown and the control *LacZ* dsRNA. Statistical analysis was performed by comparison to *LacZ* dsRNA-transfected cells. $^{**}P < 0.01$, $^{***}P < 0.001$.

an expanded polyQ tract. In summary, in *Drosophila* cells we saw dsRNA-mediated gene-specific, but not allele-specific, inhibition of transcripts encoding a truncated version of the human AR with different trinucleotide repeat lengths. In human cells, small dsRNAs specifically inhibited similar truncated *ar*-containing transcripts and, further, this inhibition protected cells from the cytotoxic effects associated with the accumulation of truncated AR protein containing an expanded polyQ tract.

In invertebrates such as *C.elegans* and *Drosophila* dsRNA-mediated RNAi is most effectively triggered by RNA duplexes of over ~80 nt. However, several recent studies have shown that the larger input dsRNA is cleaved into fragments of ~21–25 nt that act as guides for the enzymatic complex required for the degradation of the target mRNA (53,58,59,61,63,67,68). It is this observation which may explain our detection of gene-specific, but not allele-specific, inhibition as cleavage of the *arcag26ar*, *arcag43ar* or *arcag106ar* dsRNAs to dsRNAs of ~21–25 nt will likely result in a loss of any duplex that distinguishes these from one another. This obviously has significance for the application of this methodology to dominant genetic disorders where the only distinguishing feature is a trinucleotide expansion. In the case of X-linked SBMA, which is mono-allelic, this is potentially less of an issue though androgen insensitivity may be exacerbated, but where loss of function of the normal allele may have a broader clinical impact, this observation will need to be considered. Though the *arcag$_n$ar* dsRNAs did not confer allele specificity, the addition of larger *ar* flanking sequences did protect against the non-specific effects seen when dsRNAs generated from oligoribonucleotides with 27 CAG repeats were used. There are several possible reasons for this, one is that the CAG repeat results in a complex secondary folding that generates a toxic duplex; however, we saw no overt cytotoxicity and no effect on *gapdh-1* mRNA levels (data not shown). Alternatively, there is the potential that the small dsRNAs generated from the *cag27* and *arcag27* dsRNAs can target a broader ranger of transcripts that contain a small repeat, resulting in decreased expression from the parental plasmid (pAct.GFP) as well as the transcripts we were aiming to target. However, in both cases the *cag27* and the *arcag27* dsRNAs did induce a greater inhibition of the *arcag$_n$ar* transcripts than the parental plasmid suggesting at least a level of targeted degradation was occurring.

Sequence-specific dsRNA mediated interference of gene expression has only recently been observed in mammalian cell culture systems (6,7). We have now shown that the same mechanism has the potential to specifically target a transcript associated with a dominant genetic disorder and to rescue the adverse cellular changes associated with the expression of a transcript containing a trinucleotide expansion. Though the inhibition mediated in this study used a transient co-transfection system, there is evidence that the same small dsRNA methodology can be used to inhibit endogenous gene expression (6; and N.J.Caplen, unpublished data). As yet, we know little about the optimal characteristics of the small dsRNAs that mediate this

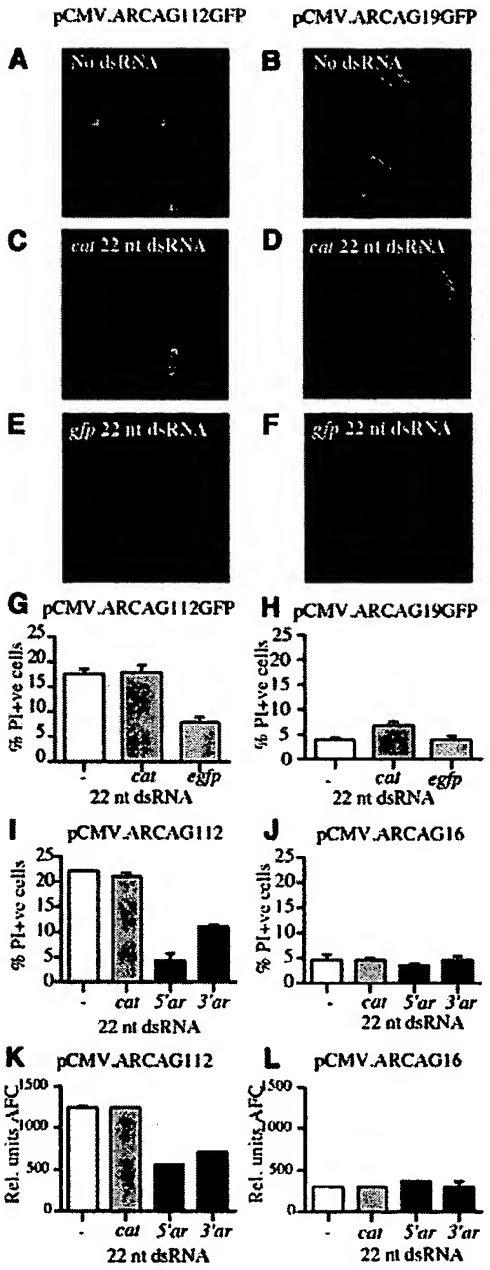

Figure 5. Specific inhibition of arcag$_n$ar transcripts by small dsRNAs in human HEK-293T cells and rescue of polyQ-induced cytotoxicity. (A–H) HEK-293T cells (1×10^6) were transfected with pCMV.ARCAG112GFP (A, C, E and G) or pCMV.ARCAG19GFP (B, D, F and H) and dsRNAs corresponding to *cat* (C, D, G and H) or *gfp* (E–H) and were assayed for GFP expression and general cell morphology by deconvolution microscopy (A–F) (GFP fluorescence is in green and nuclei are counter stained in blue using Hoeschst staining) or cell death by PI staining (G and H; data are shown as means ± SD, $n = 3$) 72 h after initiation of transfection. (I and J) HEK-293T cells (1×10^6) were transfected with pCMV.ARCAG112 (I and K) or pCMV.ARCAG16 (J and L) and dsRNAs corresponding to *cat* and two different regions of the human AR (*5'ar* and *3'ar*); cells were assayed 72 h after initiation of transfection for cell death by PI staining (I and J) and caspase-3 activity (relative fluorescence units of AFC released/min/μg protein) (K and L). Data shown are means ± SD from three independent transfections.

response in mammalian cells or the optimal nature of the target mRNA. All three of the experimental 22 nt dsRNAs used in this study inhibited expression of the target transcript, though we saw some variation in the degree of rescue mediated by the two *ar* dsRNAs, with the *5'ar* dsRNA proving more effective than the *3'ar* dsRNA. Both of these dsRNAs have similar sequence complexity and structure. However, the *5'ar* dsRNA targeted a region overlapping the start of the *ar* open-reading frame ~160 nt from the start of the CAG tract whereas the *3'ar* dsRNA targeted a sequence ~90 nt downstream of the CAG tract and it may be these features of the target sequence that explain the slight variations we observed. We are currently conducting studies to determine more precisely the parameters that affect interference by small dsRNAs in mammalian cells and importantly we now need to determine if this mechanism is also present in a wider range of cell types than have been tested so far, including neuronal cells. This study demonstrates for the first time the feasibility of targeting a disease-associated transcript by dsRNA-mediated RNAi. As our understanding of the mechanism of RNAi improves it is now increasingly likely that this methodology could be developed not only as an effective reverse genetics tool in a wide range of species but also as a gene therapy approach.

MATERIALS AND METHODS

Plasmids and generation of dsRNA

The construction of pAct.GFP and pAct.CAT have been previously described by Caplen *et al.* (51). The plasmids pAct.ARCAG26GFP, pAct.ARCAG43GFP and pAct.ARCAG106GFP were constructed by insertion of a previously constructed cassette containing 178 bp (nucleotides 524–702) and 207 bp (nucleotides 1345–1552) of the human AR 5′ and 3′ of 112 CAG repeats in frame with *egfp* (Clontech) downstream of the *Drosophila* actin 5c promoter in pRact.Adh (L.Cherabas, Indiana University, Bloomington, IN). Clones were screened for contraction or expansions of the CAG repeat during cloning and three clones with 26, 43 and 106 CAG trinucleotide repeats were identified (Fig. 1). The pAR$_{(N)}$ constructs, pCMV-ARCAG16 and pCMV-ARCAG112 have been described previously by Merry *et al.* (23). The pCMV-ARCAG19GFP and pCMV-ARCAG112.GFP plasmids were generated by sub-cloning *Xba*I–*Eco*R1 fragments from pAR$_{(N)}$ into pEGFP-N1 (Clontech) generating an in-frame fusion cDNA consisting of a truncated form of the human AR and enhanced GFP (EGFP).

Single-stranded, gene-specific sense and antisense RNA oligomers corresponding to the β-galactosidase (*LacZ*) gene, the CAT (*cat*) gene, the GFP protein (*gfp*) gene and the human *ar* gene (sequences shown in Table 1) were synthesized using 2′-*O*-(triisopropyl)silyloxymethyl chemistry by Xeragon AG. Sense and antisense oligoribonucleotides consisting of 27 CAG trinucleotides and 27 CAG trinucleotide repeats plus 21 nt from the 5′ region of the human AR immediately adjacent to the CAG tract (see Table 1 for sequences) were also chemically synthesized. To form dsRNA molecules 100 μg of each complementary single-stranded RNA (ssRNA) oligomer in 10 mM Tris–Cl pH 7.0, 20 mM NaCl (total volume 300 μl) was heat denatured at 95°C in a water bath and then allowed to anneal slowly (in the water bath) for ~18 h. The dsRNAs

were ethanol precipitated and re-suspended in RNase free water at a concentration of ~0.5 mg/ml. Double-stranded RNA molecules corresponding to *arcag26ar*, *arcag43ar* and *arcag106ar* were generated by sub-cloning each cassette as an *Eco*RI–*Bss*HII fragment into pCMV-BK (Stratagene). Sense and antisense RNAs were synthesized for each cassette using standard T3 and T7 RNA polymerase *in vitro* transcription reactions (Megascript; Ambion) after preparation of a *Pvu*II–*Nhe*I fragment which included non-homologous plasmid sequences. Double-stranded RNAs were generated as above; after precipitation flanking ssRNAs derived from non-homologous plasmid sequences were removed using Ribonuclease T1 in 0.3 M NaCl. The integrity and change in mobility of all of the dsRNAs were confirmed by gel electrophoresis.

Cell culture and nucleic acid transfections

S2 cells were grown in DES™ Medium (Invitrogen) supplemented with 10% fetal bovine serum (FBS). Cells were passaged every 2–3 days to maintain exponential growth. S2 cells were transfected using either the cationic lipid CellFectin (Life Technologies) using an adaptation of the manufacturer's protocol. Briefly, cells were seeded and allowed to settle overnight, nucleic acid (plasmid and/or dsRNA) was complexed with lipid at a weight to weight ratio of 1:6 in DES medium without supplementation. The complex was incubated at room temperature for 15 min and then added to cells from which normal growth medium had been removed. After overnight incubation an equal volume of DES medium plus 20% FBS was added to the cell/lipoplex mixture. HEK-293T cells were maintained in DMEM medium (Gibco BRL) supplemented with 10% FBS. Cells were plated at a density of 60% in six-well culture dishes the day prior to transfection. Transfection with 1 μg of DNA was carried out using Lipofectamine Plus following the manufacturer's protocol (Gibco BRL) plus 0.5 μg of dsRNA.

Transgene expression

GFP expression was assessed by FACS analysis or deconvolution microscopy. For FACS analysis (FacsCaliber; Becton Dickinson) pAct.CAT-transfected cells were used to gate for forward scatter and side scatter. The percentage of GFP-positive cells was determined by gating against pAct.CAT-transfected cells with M1 falling between 10 or 20 and 9910 (FL-1), the geometric mean was used as a measure of the relative intensity of fluorescence. 10 000 non-gated events were acquired for each sample. For deconvolution microscopy S2 and 293 cells were either spun onto slides or grown on coverslips or two-well chamber-slides (Nunc). At the indicated times the cells were fixed in 4% paraformaldehyde for 15 min at room temperature then washed three times in PBS. All cells were stained with 1 μg/ml Hoechst 33342 (Sigma) in PBS for 10 min. Deconvolved images were produced on an Olympus microscope using a 60× water-immersion objective and Deltavision software (Applied Precision) on a Silicon Graphics workstation. Multiple optical sections 0.2 μm thick were analyzed.

To directly measure the toxicity of expanded polyglutamine in the context of truncated AR we employed a FACS-based survival assay. HEK-293T cells were harvested with trypsin, gently pelleted by centrifugation and resuspended in PBS with 0.5% serum ice at a concentration 10^6/ml. The cells were stained with 1 μg/ml propidium iodide (PI) (Sigma), gently vortexed and incubated for 15 min at room temperature in the dark. 50 000 non-gated events were acquired for each sample (Beckman Coulter XL instrument and software package used for analysis). Results are expressed as a percentage of PI-negative (viable) cells (FL-2 channel) relative to total GFP-positive (transfected) cells (FL-1 channel).

CAT expression was assessed using an ELISA-based method (Roche Biochemicals). Total protein was determined using the Bradford method and the micro-assay protocol adapted for use in 96-well micro-titer plates (Bio-Rad). Absorption readings (A_{595}) were converted to absolute amounts using a bovine serum albumin (Sigma) (0.625–10 μg) standard curve after subtraction of background values. Statistical analyses were conducted using an unpaired *t*-test (SatView) for comparison of groups. The null hypothesis was rejected at $P > 0.05$.

RNA analysis

Total RNA was isolated using GTC extraction and poly(A)$^+$ RNA selected using Oligo dT cellulose (Ambion). The poly(A)$^+$ RNA was subjected to DNase digestion to remove any trace contamination of plasmid DNA and analyzed by electrophoresis (1.2% agarose, 1× MOPS, 5.0% formaldehyde), northern blot transfer and hybridization (Ambion) at 42°C with ^{32}P-labeled random-primed DNA probes. A human AR gene-specific probe (148 bp) was prepared by PCR amplification using pCMV.AR112GFP as template and the following oligonucleotides (*ar* forward, 5′-gga agt gca gtt agg gct g-3′; *ar* reverse, 5′-cag cag cag caa act ggc g-3′). The *gfp*, and *Drosophila gapdh-1* DNA probes have been previously described by Caplen *et al.* (51). In all cases, filters were washed at high stringency (0.2× SSC) and subjected to autoradiography. The intensities of the hybridization signals were obtained using a Fuji BAS1500 phosphoimager (Fuji Photo Film Co.) and pixel densities calculated using Image reader 1.4 and ImageGauge 3.0 (Fuji Photo Film Co.).

Caspase-3 activity

To quantitate caspase-3 activity, 10^6 cells were washed with cold PBS and harvested by scraping at the indicated times post-transfection. The cells were pelleted at 400 *g* for 5 min then re-suspended in lysis buffer (10 mM Tris, pH 7.3, 10 mM NaH_2PO_4, 150 mM NaCl, 1% Triton X-100). Protein concentration was determined using the DC protein assay reagent (Bio Rad). 100 mg of cell extract was incubated with 50 mM of fluorometric substrate Asp–Glu–Val–Asp–7-amino-4-trifluoromethylcoumarin (DEVD–AFC) (ApoTarget kit; Biosource International) in a total volume of 100 μl of reaction buffer (10 mM HEPES, pH 7.4, 50 mM NaCl, 0.5 mM EDTA, 0.1% CHAPS, 10% glycerol, 10 mM DTT) in the dark for 1 h at 37°C. DEVDase activity was determined by measuring the liberation of AFC using a Cytofluor II multiwell plate reader (PerSeptive Biosystems) with excitation and emission wavelengths of 420 and 520 nm, respectively. Caspase-3 specificity was established by blocking activity with the inhibitor DEVD–CHO at 10 mM.

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REFERENCES

1. Cogoni,C. and Macino,G. (2000) Post-transcriptional gene silencing across kingdoms. *Curr. Opin. Genet. Dev.*, **10**, 638–643.
2. Fire,A., Xu,S., Montgomery,M.K., Kostas,S.A., Driver,S.E. and Mello,C.C. (1998) Potent and specific genetic interference by double-stranded RNA in *Caenorhabditis elegans*. *Nature*, **391**, 806–811.
3. Montgomery,M.K., Xu,S. and Fire,A. (1998) RNA as a target of double-stranded RNA-mediated genetic interference in *Caenorhabditis elegans*. *Proc. Natl Acad. Sci. USA*, **95**, 15502–15507.
4. Wianny,F. and Zernicka-Goetz,M. (2000) Specific interference with gene function by double-stranded RNA in early mouse development. *Nat. Cell Biol.*, **2**, 70–75.
5. Svoboda,P., Stein,P., Hayashi,H. and Schultz,R.M. (2000) Selective reduction of dormant maternal mRNAs in mouse oocytes by RNA interference. *Development*, **127**, 4147–4156.
6. Elbashir,S.M., Harborth,J., Lendeckel,W., Yalcin,A., Weber,K. and Tuschl,T. (2001) Duplexes of 21-nucleotide RNAs mediate RNA interference in cultured mammalian cells. *Nature*, **411**, 494–498.
7. Caplen,N.J., Parrish,S., Imani,F., Fire,A. and Morgan,R.A. (2001) Specific inhibition of gene expression by small double-stranded RNAs in invertebrate and vertebrate systems. *Proc. Natl Acad. Sci. USA*, **98**, 9742–9747.
8. Kuwabara,P.E. and Coulson,A. (2000) RNAi—prospects for a general technique for determining gene function. *Parasitol. Today*, **16**, 347–349.
9. Kennerdell,J.R. and Carthew,R.W. (1998) Use of dsRNA-mediated genetic interference to demonstrate that *frizzled* and *frizzled 2* act in the wingless pathway. *Cell*, **95**, 1017–1026.
10. Misquitta,L. and Paterson,B.M. (1999) Targeted disruption of gene function in *Drosophila* by RNA interference (RNA-i): a role for *nautilus* in embryonic somatic muscle formation. *Proc. Natl Acad. Sci. USA*, **96**, 1451–1456.
11. Jen,K.Y. and Gewirtz,A.M. (2000) Suppression of gene expression by targeted disruption of messenger RNA: available options and current strategies. *Stem Cells*, **18**, 307–319.
12. Cummings,C.J. and Zoghbi,H.Y. (2000) Fourteen and counting: unraveling trinucleotide repeat diseases. *Hum. Mol. Genet.*, **9**, 909–916.
13. Lieberman,A.P. and Fischbeck,K.H. (2000) Triplet repeat expansion in neuromuscular disease. *Muscle Nerve*, **23**, 843–850.
14. Paulson,H.L. (1999) Protein fate in neurodegenerative proteinopathies: polyglutamine diseases join the (mis)fold. *Am. J. Hum. Genet.*, **64**, 339–345.
15. Li,M., Miwa,S., Kobayashi,Y., Merry,D.E., Yamamoto,M., Tanaka,F., Doyu,M., Hashizume,Y., Fischbeck,K.H. and Sobue,G. (1998) Nuclear inclusions of the androgen receptor protein in spinal and bulbar muscular atrophy. *Ann. Neurol.*, **44**, 249–254.
16. Li,M., Nakagomi,Y., Kobayashi,Y., Merry,D.E., Tanaka,F., Doyu,M., Mitsuma,T., Hashizume,Y., Fischbeck,K.H. and Sobue,G. (1998) Nonneural nuclear inclusions of androgen receptor protein in spinal and bulbar muscular atrophy. *Am. J. Pathol.*, **153**, 695–701.
17. DiFiglia,M., Sapp,E., Chase,K.O., Davies,S.W., Bates,G.P., Vonsattel,J.P. and Aronin,N. (1997) Aggregation of huntingtin in neuronal intranuclear inclusions and dystrophic neurites in brain. *Science*, **277**, 1990–1993.
18. Davies,S.W., Turmaine,M., Cozens,B.A., DiFiglia,M., Sharp,A.H., Ross,C.A., Scherzinger,E., Wanker,E.E., Mangiarini,L. and Bates,G.P. (1997) Formation of neuronal intranuclear inclusions underlies the neurological dysfunction in mice transgenic for the HD mutation. *Cell*, **90**, 537–548.
19. Reddy,P.H., Williams,M., Charles,V., Garrett,L., Pike-Buchanan,L., Whetsell,W.O.,Jr, Miller,G. and Tagle,D.A. (1998) Behavioural abnormalities and selective neuronal loss in HD transgenic mice expressing mutated full-length HD cDNA. *Nat. Genet.*, **20**, 198–202.
20. Abel,A., Walcott,J., Woods,J., Duda,J. and Merry,D.E. (2001) Expression of expanded repeat androgen receptor produces neurologic disease in transgenic mice. *Hum. Mol. Genet.*, **10**, 107–116.
21. Abdullah,A., Trifiro,M.A., Panet-Raymond,V., Alvarado,C., de Tourreil,S., Frankel,D., Schipper,H.M. and Pinsky,L. (1998) Spinobulbar muscular atrophy: polyglutamine-expanded androgen receptor is proteolytically resistant *in vitro* and processed abnormally in transfected cells. *Hum. Mol. Genet.*, **7**, 379–384.
22. Butler,R., Leigh,P.N., McPhaul,M.J. and Gallo,J.M. (1998) Truncated forms of the androgen receptor are associated with polyglutamine expansion in X-linked spinal and bulbar muscular atrophy. *Hum. Mol. Genet.*, **7**, 121–127.
23. Merry,D.E., Kobayashi,Y., Bailey,C.K., Taye,A.A. and Fischbeck,K.H. (1998) Cleavage, aggregation and toxicity of the expanded androgen receptor in spinal and bulbar muscular atrophy. *Hum. Mol. Genet.*, **7**, 693–701.
24. Saudou,F., Finkbeiner,S., Devys,D. and Greenberg,M.E. (1998) Huntingtin acts in the nucleus to induce apoptosis but death does not correlate with the formation of intranuclear inclusions. *Cell*, **95**, 55–66.
25. Klement,I.A., Skinner,P.J., Kaytor,M.D., Yi,H., Hersch,S.M., Clark,H.B., Zoghbi,H.Y. and Orr,H.T. (1998) Ataxin-1 nuclear localization and aggregation: role in polyglutamine-induced disease in SCA1 transgenic mice. *Cell*, **95**, 41–53.
26. Simeoni,S., Mancini,M.A., Stenoien,D.L., Marcelli,M., Weigel,N.L., Zanisi,M., Martini,L. and Poletti,A. (2000) Motoneuronal cell death is not correlated with aggregate formation of androgen receptors containing an elongated polyglutamine tract. *Hum. Mol. Genet.*, **9**, 133–144.
27. Ikeda,H., Yamaguchi,M., Sugai,S., Aze,Y., Narumiya,S. and Kakizuka,A. (1996) Expanded polyglutamine in the Machado-Joseph disease protein induces cell death *in vitro* and *in vivo*. *Nat. Genet.*, **13**, 196–202.
28. Scherzinger,E., Lurz,R., Turmaine,M., Mangiarini,L., Hollenbach,B., Hasenbank,R., Bates,G.P., Davies,S.W., Lehrach,H. and Wanker,E.E. (1997) Huntingtin-encoded polyglutamine expansions form amyloid-like protein aggregates *in vitro* and *in vivo*. *Cell*, **90**, 549–558.
29. Lunkes,A. and Mandel,J.L. (1998) A cellular model that recapitulates major pathogenic steps of Huntington's disease. *Hum. Mol. Genet.*, **7**, 1355–1361.
30. Scherzinger,E., Sittler,A., Schweiger,K., Heiser,V., Lurz,R., Hasenbank,R., Bates,G.P., Lehrach,H. and Wanker,E.E. (1999) Self-assembly of polyglutamine-containing huntingtin fragments into amyloid-like fibrils: implications for Huntington's disease pathology. *Proc. Natl Acad. Sci. USA*, **96**, 4604–4609.
31. Marsh,J.L., Walker,H., Theisen,H., Zhu,Y.Z., Fielder,T., Purcell,J. and Thompson,L.M. (2000) Expanded polyglutamine peptides alone are intrinsically cytotoxic and cause neurodegeneration in *Drosophila*. *Hum. Mol. Genet.*, **9**, 13–25.
32. Goldberg,Y.P., Nicholson,D.W., Rasper,D.M., Kalchman,M.A., Koide,H.B., Graham,R.K., Bromm,M., Kazemi-Esfarjani,P., Thornberry,N.A., Vaillancourt,J.P. *et al.* (1996) Cleavage of huntingtin by apopain, a proapoptotic cysteine protease, is modulated by the polyglutamine tract. *Nat. Genet.*, **13**, 442–449.
33. Wellington,C.L., Ellerby,L.M., Hackam,A.S., Margolis,R.L., Trifiro,M.A., Singaraja,R., McCutcheon,K., Salvesen,G.S., Propp,S.S., Bromm,M. *et al.* (1998) Caspase cleavage of gene products associated with triplet expansion disorders generates truncated fragments containing the polyglutamine tract. *J. Biol. Chem.*, **273**, 9158–9167.
34. Kobayashi,Y., Miwa,S., Merry,D.E., Kume,A., Mei,L., Doyu,M. and Sobue,G. (1998) Caspase-3 cleaves the expanded androgen receptor protein of spinal and bulbar muscular atrophy in a polyglutamine repeat length-dependent manner. *Biochem. Biophys. Res. Commun.*, **252**, 145–150.
35. Ellerby,L.M., Hackam,A.S., Propp,S.S., Ellerby,H.M., Rabizadeh,S., Cashman,N.R., Trifiro,M.A., Pinsky,L., Wellington,C.L., Salvesen,G.S. *et al.* (1999) Kennedy's disease: caspase cleavage of the androgen receptor is a crucial event in cytotoxicity. *J. Neurochem.*, **72**, 185–195.
36. Hackam,A.S., Yassa,A.S., Singaraja,R., Metzler,M., Gutekunst,C.A., Gan,L., Warby,S., Wellington,C.L., Vaillancourt,J., Chen,N. *et al.* (2000) Huntingtin interacting protein 1 induces apoptosis via a novel caspase-dependent death effector domain. *J. Biol. Chem.*, **275**, 41299–41308.
37. Wellington,C.L., Singaraja,R., Ellerby,L., Savill,J., Roy,S., Leavitt,B., Cattaneo,E., Hackam,A., Sharp,A., Thornberry,N. *et al.* (2000) Inhibiting caspase cleavage of huntingtin reduces toxicity and aggregate formation in neuronal and nonneuronal cells. *J. Biol. Chem.*, **275**, 19831–19838.
38. Cummings,C.J., Mancini,M.A., Antalffy,B., DeFranco,D.B., Orr,H.T. and Zoghbi,H.Y. (1998) Chaperone suppression of aggregation and altered subcellular proteasome localization imply protein misfolding in SCA1. *Nat. Genet.*, **19**, 148–154.
39. Warrick,J.M., Chan,H.Y., Gray-Board,G.L., Chai,Y., Paulson,H.L. and Bonini,N.M. (1999) Suppression of polyglutamine-mediated neurodegeneration in *Drosophila* by the molecular chaperone HSP70. *Nat. Genet.*, **23**, 425–428.

40. Satyal,S.H., Schmidt,E., Kitagawa,K., Sondheimer,N., Lindquist,S., Kramer,J.M. and Morimoto,R.I. (2000) Polyglutamine aggregates alter protein folding homeostasis in *Caenorhabditis elegans*. *Proc. Natl Acad. Sci. USA*, **97**, 5750–5755.
41. Kazemi-Esfarjani,P. and Benzer,S. (2000) Genetic suppression of polyglutamine toxicity in *Drosophila*. *Science*, **287**, 1837–1840.
42. Sanchez,I., Xu,C.J., Juo,P., Kakizaka,A., Blenis,J. and Yuan,J. (1999) Caspase-8 is required for cell death induced by expanded polyglutamine repeats. *Neuron*, **22**, 623–633.
43. Ona,V.O., Li,M., Vonsattel,J.P., Andrews,L.J., Khan,S.Q., Chung,W.M., Frey,A.S., Menon,A.S., Li,X.J., Stieg,P.E. *et al.* (1999) Inhibition of caspase-1 slows disease progression in a mouse model of Huntington's disease. *Nature*, **399**, 263–267.
44. Agrawal,S. and Kandimalla,E.R. (2000) Antisense therapeutics: is it as simple as complementary base recognition? *Mol. Med. Today*, **6**, 72–81.
45. Muotri,A.R., da Veiga Pereira,L., dos Reis Vasques,L. and Menck,C.F. (1999) Ribozymes and the anti-gene therapy: how a catalytic RNA can be used to inhibit gene function. *Gene*, **237**, 303–310.
46. Sun,L.Q., Cairns,M.J., Saravolac,E.G., Baker,A. and Gerlach,W.L. (2000) Catalytic nucleic acids: from lab to applications. *Pharmacol. Rev.*, **52**, 325–347.
47. Montgomery,R.A. and Dietz,H.C. (1997) Inhibition of fibrillin 1 expression using U1 snRNA as a vehicle for the presentation of antisense targeting sequence. *Hum. Mol. Genet.*, **6**, 519–525.
48. Nellemann,C., Abell,K., Norremolle,A., Lokkegaard,T., Naver,B., Ropke,C., Rygaard,J., Sorensen,S.A. and Hasholt,L. (2000) Inhibition of Huntington synthesis by antisense oligodeoxynucleotides. *Mol. Cell. Neurosci.*, **16**, 313–323.
49. O'Neill,B., Millington-Ward,S., O'Reilly,M., Tuohy,G., Kiang,A.S., Kenna,P.F., Humphries,P. and Farrar,G.J. (2000) Ribozyme-based therapeutic approaches for autosomal dominant retinitis pigmentosa. *Invest. Ophthalmol. Vis. Sci.*, **41**, 2863–2869.
50. Millington-Ward,S., O'Neill,B., Tuohy,G., Al-Jandal,N., Kiang,A.S., Kenna,P.F., Palfi,A., Hayden,P., Mansergh,F., Kennan,A. *et al.* (1997) Strategems *in vitro* for gene therapies directed to dominant mutations. *Hum. Mol. Genet.*, **6**, 1415–1426.
51. Caplen,N.J., Fleenor,J., Fire,A. and Morgan,R.A. (2000) dsRNA-mediated gene silencing in cultured *Drosophila* cells: a tissue culture model for the analysis of RNA interference. *Gene*, **252**, 95–105.
52. Clemens,J.C., Worby,C.A., Simonson-Leff,N., Muda,M., Maehama,T., Hemmings,B.A. and Dixon,J.E. (2000) Use of double-stranded RNA interference in *Drosophila* cell lines to dissect signal transduction pathways. *Proc. Natl Acad. Sci. USA*, **97**, 6499–6503.
53. Hammond,S.M., Bernstein,E., Beach,D. and Hannon,G.J. (2000) An RNA-directed nuclease mediates post-transcriptional gene silencing in *Drosophila* cells. *Nature*, **404**, 293–296.
54. Jackson,G.R., Salecker,I., Dong,X., Yao,X., Arnheim,N., Faber,P.W., MacDonald,M.E. and Zipursky,S.L. (1998) Polyglutamine-expanded human huntingtin transgenes induce degeneration of *Drosophila* photoreceptor neurons. *Neuron*, **21**, 633–642.
55. Warrick,J.M., Paulson,H.L., Gray-Board,G.L., Bui,Q.T., Fischbeck,K.H., Pittman,R.N. and Bonini,N.M. (1998) Expanded polyglutamine protein forms nuclear inclusions and causes neural degeneration in *Drosophila*. *Cell*, **93**, 939–949.
56. Bonini,N.M. (1999) A genetic model for human polyglutamine-repeat disease in *Drosophila melanogaster*. *Phil. Trans. R. Soc. Lond. B. Biol. Sci.*, **354**, 1057–1060.
57. Martindale,D., Hackam,A., Wieczorek,A., Ellerby,L., Wellington,C., McCutcheon,K., Singaraja,R., Kazemi-Esfarjani,P., Devon,R., Kim,S.U. *et al.* (1998) Length of huntingtin and its polyglutamine tract influences localization and frequency of intracellular aggregates. *Nat. Genet.*, **18**, 150–154.
58. Parrish,S., Fleenor,J., Xu,S., Mello,C. and Fire,A. (2000) Functional anatomy of a dsRNA trigger. Differential requirement for the two trigger strands in RNA interference. *Mol. Cell*, **6**, 1077–1087.
59. Zamore,P.D., Tuschl,T., Sharp,P.A. and Bartel,D.P. (2000) RNAi: double-stranded RNA directs the ATP-dependent cleavage of mRNA at 21 to 23 nucleotide intervals. *Cell*, **101**, 25–33.
60. Tuschl,T., Zamore,P.D., Lehmann,R., Bartel,D.P. and Sharp,P.A. (1999) Targeted mRNA degradation by double-stranded RNA *in vitro*. *Genes Dev.*, **13**, 3191–3197.
61. Yang,D., Lu,H. and Erickson,J.W. (2000) Evidence that processed small dsRNAs may mediate sequence-specific mRNA degradation during RNAi in *Drosophila* embryos. *Curr. Biol.*, **10**, 1191–1200.
62. Clemens,M.J. and Elia,A. (1997) The double-stranded RNA-dependent protein kinase PKR: structure and function. *J. Interferon Cytokine Res.*, **17**, 503–524.
63. Bernstein,E., Caudy,A.A., Hammond,S.M. and Hannon,G.J. (2001) Role for a bidentate ribonuclease in the initiation step of RNA interference. *Nature*, **409**, 363–366.
64. Vance,V. and Vaucheret,H. (2001) RNA silencing in plants-defense and counterdefense. *Science*, **292**, 2277–2280.
65. Matzke,M., Matzke,A.J.M. and Kooter,J.M. (2001) RNA: Guiding gene silencing. *Science*, **293**, 1080–1083.
66. Barstead,R. (2001) Genome-wide RNAi. *Curr. Opin. Chem. Biol.*, **5**, 63–66.
67. Hamilton,A.J. and Baulcombe,D.C. (1999) A species of small antisense RNA in posttranscriptional gene silencing in plants. *Science*, **286**, 950–952.
68. Elbashir,S.M., Lendeckel,W. and Tuschl,T. (2001) RNA interference is mediated by 21- and 22-nucleotide RNAs. *Genes Dev.*, **15**, 188–200.